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- (54) **DNA-molecules coding for FMDH control regions and structured gene for a protein having FMDH-activity and their uses.**

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Description

The present invention is directed to a DNA fragment comprising a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having format dehydrogenase (FMDH) activity, recombinant vectors containing said fragment, microorganisms containing said vectors and a process for producing a substance using the microorganisms according to the present invention.

During the last decade, several yeast strains were isolated which are able to utilize methanol as an only carbon and energy source. Until recently the studies were limited to the enzymatic level and concerned mainly two species, namely *Hansenula polymorpha* and *Candida boidinii*.

The enzymatic studies revealed that in methylotrophic yeasts methanol is oxidised via formaldehyde and formate to CO₂ by methanol oxidase (MOX), formaldehyde dehydrogenase (FMD) and formate dehydrogenase (FMDH), respectively. H₂O₂ which is generated during the first oxidation step is degraded by catalase. C1 compound is assimilated by transketolase reaction of xylulose-5-(P) and formaldehyde, the latter being derived from the dissimilatory pathway. The reaction is catalysed by dihydroxyacetone synthase (DHAS).

Growth of methylotrophic yeast on methanol is accompanied by changes in total protein composition. There are 3 major and about 5 minor proteins newly synthesized. Further, the growth on methanol is accompanied by appearance of huge peroxisomes. These organelles bear some of the key enzymes involved in methanol metabolism, namely, MOX, DHAS and catalase (1). The other two methanol enzymes FMD and FMDH, are cytoplasmic proteins. In methanol grown cells, the enzymes FMDH, MOX, and DHAS constitute up to 40% of total cell protein. The methanol utilisation pathway is highly compartmentalised and the integration of these reactions is very complex.

The methanol dissimilatory enzymes are regulated by glucose catabolite repression/derepression mechanism (2). Methanol has an additional inductive effect increasing the expression level by the factor of 2-3. In *H. polymorpha*, assimilatory DHAS enzyme follows this general regulation scheme, however, during growth on limiting amounts of glucose, derepression, an additional post transcriptional mechanism, plays a role in the regulation.

Recently, 3 genes encoding peroxisomal enzymes were cloned from *H. polymorpha* and *Pichia pastoris* and the analysis of nucleotide sequences of MOX genes from *H. polymorpha* (3) and *P. pastoris* (4) and DAS gene, which encodes DHAS from *H. polymorpha* (5) revealed that a cleavable signal sequence is not required for the transport of MOX and DHAS into the peroxisome.

The promoters of some methanol genes are very efficient and their way of regulation is favourable to the industrial application. The expression of foreign proteins can be enhanced and placed under stringent control. The large amounts of proteins (MOX, DHAS) thus produced by methylotrophic yeast are stored in the peroxisomes. The understanding of this mechanism will help to solve some problems of the stability of foreign proteins in yeast.

In the field of industrial biotechnology, there is a need for microbiological regulation systems by which large amounts of a particularly desired protein can be produced under stringent control. Although there are already promoter/terminator systems available which can be used in genetic engineering systems for controlling the amount of proteins to be produced, there is still a strong need for further regulatory systems to be available since it has turned out that, in biological systems, it is advantageous to provide more systems so that the most effective one can be chosen. The present systems are far from being efficient, especially when stringent regulation and high mitotic stability is required.

It was, therefore, an object of the present invention to provide a more effective and a very easily controllable regulatory system.

The advantage of the present invention is given by providing a DNA fragment, which comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the *Hansenula polymorpha* genome, wherein the FMDH gene is located on a 3.5 kb *Bam*HI-*Hind*III fragment.

To start more comprehensive studies on basic research and biotechnological aspects of methanol utilisation, the gene encoding the cytoplasmic methanol key enzyme FMDH was cloned. The sequence of this 1020 bp long gene and its regulatory regions have been cloned. FMDH is regulated at transcriptional level by glucose catabolite repression/depression/methanol induction mechanism.

The DNA-fragment according to this invention is extremely useful in the biotechnology industry because of the above discussed characteristic that the expression of foreign proteins can be enhanced and placed under Stringent control.

The DNA fragments according to the present invention may be modified by recombinant DNA technology techniques as known in the art, resulting in a modified promoter, which still retains its promoter function.

A preferred embodiment of the DNA-molecule of this invention is shown in Fig. 5.

Examples for the use of the FMDH regulatory sequences of the present invention are combinations of said DNA sequences with foreign genes encoding hepatitis B virus S1-S2-S antigen and hepatitis B virus S antigen α -amylase from *S. castelli* and glucoamylase from *S. castelli* or invertase from *Saccharomyces cerevisiae*.

The DNA- fragments of this invention may further be combined to DNA-sequences which are coding for secretory signals, such as *Hansenula polymorpha* membrane translocation signals, preferably those from peroxisomal proteins, methanol oxidase and dihydroxyacetone synthase, *Schwanniomyces castelli* α -amylase and glucoamylase signals, or *Saccharomyces cerevisiae* α -factor and invertase signals.

Preparation of the DNA-fragments coding for control regions and the structural gene for protein having FMDH activity may be obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

Recombinant vectors can be prepared which contain the DNA fragments according to this invention either as such, coding for the regulatory regions and/or structural genes for FMDH protein and may be combined to further DNA sequences as discussed above. Recombinant vectors for the purposes of transferring DNA sequences into an expression system are commonly used in the art and may be properly chosen. For example, the λ Charon 4A phage may carry the described DNA- fragments.

As micro-organisms which are suitable for the expression of the desired genes also may be selected from known micro-organisms in the art which are adapted for recombinant DNA technologies. Micro-organisms, however, who are able to tolerate high concentrations of foreign proteins are preferred.

Most preferred are micro-organisms of the genera *Candida*, *Hansenula* or *Pichia*.

The mentioned micro-organisms are able to produce the desired substances either by integration of the DNA-molecules of this invention into the chromosom of the micro-organism or by maintaining the DNA-molecules on an extra chromosomal DNA-molecule via episomal vectors.

The proteins coded by foreign genes combined to the DNA-fragments of the present invention and being produced by the transformed micro-organisms can be obtained by culturing said micro-organisms in a manner known in the art and recovering the proteins as is also standard knowledge in the art.

The invention is now presented, in a more detailed manner, by the following specification and figures. The figures show:

Figure 1:

Analysis of protein crude extracts and *in vitro* translation products by SDS-polyacrylamid gel electrophoresis.

Lanes 7-9: Coomassie Blue stained gel; protein crude extracts from induced, derepressed and uninduced cells, respectively. Lane 10, purified FMDH. Lanes 1-3: 35 S-labelled *in vitro* translation products of mRNA isolated from induced, uninduced cells and fractionated mRNA enriched in FMDH mRNA species, respectively. Lane 4; immunoprecipitation of translation products from lane 1. Lane 5; translation of hybrid-selected mRNA. Lane 6: immunoprecipitation of translation products from lane 5.

Figure 2:

Restriction map of DNA fragment encompassing the FMDH gene.

The arrow shows the direction of transcription.

Figure 3:

S1-mapping;

Lanes M1, 1, 2, 3, 4, M2, a, b, c, d, separation on alkaline agarose gel. Lanes 5, M3-separation on 6% polyacrylamide gel/8M urea. Lanes M1-M3-MW markers. Lanes 1, 2-total protection (1) of 4.1 kb Eco-RI/Hind III fragment (2) encompassing the gene. Lanes 3, 4-protection of 3'-end labelled 1.4 kb Bam HI/Hind III fragment; 3-protected band; 4-1.4 kb intact band. Lane 5-protection of 1 kb Bam HI/Pst I fragment with a single label at Bam HI site. Lanes a, b, c, d-protection of 3'-end labelled DNA fragment containing part of the gene by mRNA preparation isolated from: induced, derepressed (1% glycerol), stationary phase of 3% glucose and mid-log phase of 3% glucose cultures, respectively.

Figure 4:

Sequencing strategy - schematic representation.

DNA fragments containing the gene were subjected to Bal31 digestion and the resulting fragments subcloned into M13 and/or pUC type vectors. The fragments were sequenced by Sanger and in the case of doubts Maxam-Gilbert

- methods.
- Figure 5a: Nucleotide sequence of FMDH gene and its 5', 3' control regions.
- Figure 5b: Nucleotide sequence of FMDH gene and its 5', 3' control regions.
- Figure 5c: Nucleotide sequence of FMDH gene and its 5', 3' control regions.
- 5 Figure 6: Plasmid containing the fusion of bacterial β -lactamase gene with FMDH promoter.
- Figure 7: Plasmid containing the hepatitis S-gene; HARS - *H. polymorpha* autonomous replicating sequence; URA3 - *S. cerevisiae* gene; FMDH-promoter (-9 type promoter).
- 10 Figure 8: Western blot-stained by peroxidase/protein A method. Polyclonal antibodies (not clarified) were used in this experiment:
- Lane a : LR9 growth on methanol
- Lane i : transformant w/o S-gene
- Lanes k, l, m : transformants with S-gene grown on glucose (repression)
- 15 Lanes b, c, d, e, f, g : different transformants with S-gene grown on methanol
- Lanes n, o : 500 and 450 ng purified HSBAG, respectively
- Figure 9: Plasmid expressing α -amylase gene; symbols are the same as in Fig. 7.
- Figure 10: Growth of transformants on medium containing methanol (induction). Enzyme activity (U/ml) was measured in medium and in cells (intracellular enzyme level). The latter value was expressed as corresponding to 1 ml of medium.
- 20 Figure 11: The formation of halo after applying on the plate 50 μ l of the medium from transformants (upper row) and from control untransformed strain LR9 (lower row).

25 Strains, media, vectors:

Thermophilic, homothallic strain of *H. polymorpha* (ATCC 34438) was used. Yeast was grown at 37°C on minimal YNB medium as described (3, 5). Induction of methanol utilisation system was achieved by growth in minimal medium containing 1% methanol; growth on 3% glucose minimal medium resulted in repression of the system.

E. coli L90; C600recA, hsdM, araB, was used for transformation;

E. coli JM103, thi, strA, supE, endA, sbcB, hsdR, F'traD36, proAB, lacI, ZM15, and

E. coli KH802 gal, met, supE, were used as host for phage M13 and for λ -vector Charon 4A, respectively.

35 Plasmid DNA and RF M 13 were isolated by scaled-up alkaline minilysates methods (6) followed by CsCl ultracentrifugation.

λ -vector Charon 4A and Charon 4 recombinant clones were isolated by scaled-up plate lysate methods (6).

H. polymorpha total DNA of the size greater than 50 kb was isolated from spheroplasts as previously described (5).

40 Charon 4 *H. polymorpha* DNA library was constructed by ligating partially EcoRI digested *H. polymorpha* DNA with Charon 4 arms as described previously (5).

PolyA mRNA from *H. polymorpha* and analysis of the mRNA by an *in vitro* cell free rabbit reticulocyte system is described previously (5).

mRNA labelling: mRNA was partially fragmented by mild alkaline treatment (7) and labelled at the 5'-end with γ -³²P-ATP (Amersham).

The differential plaque filter hybridisation was performed essentially as described in (12). Recombinant phages were plated to about 3,000 pfu per plate. Plaques from each plate were blotted into a set of 5-6 replica nitro-cellulose filters (BA85, Schleicher and Schüll). The filters were hybridized to appropriate ³²P-mRNA or ³²P-DNA probes in 5 x SSPE, 50%, formamide, containing additionally 150 μ g/ml tRNA, 10 μ g/ml poly A, 5 x Denhardt's solution, 5 μ g/ml rRNA from *H. polymorpha* isolated as described in (5, 6).

S1 mapping experiments were performed essentially as described by Favarolo et al. (8). S1 nuclease from NEN at concentration 1,000 units/ml was used.

Hybrid selection technique was performed as described by Büneman et al. (9). Briefly, DNA from recombinant subclones was covalently bound to DPTE derivative of Sephacryl S-500. Total mRNA was then hybridized with DNA/S-500 matrix. mRNA species not complementary to the immobilized DNA were washed out under very stringent conditions (5, 9). Hybridized mRNA was eluted with H₂O at 100°C. Hybrid selected mRNA was then translated in cell-free system, and the translation products analyzed by immunoprecipitation as described previously.

Sequence analysis: Different overlapping fragments derived from the exonuclease Bal31 digestion of DNA fragments encompassing FMDH gene were cloned into M13 phages mp9, mp8 and into plasmid pUC12, pUC13. The subcloned fragments were sequenced by Sanger et al. (10) and Maxam-Gilbert (11) methods.

Formate dehydrogenase was purified to homogeneity from methanol grown *H. polymorpha* cells as described elsewhere. Antibodies against FMDH, denaturated form, were raised in rabbits according to standard procedures.

Identification of mRNA species encoding FMDH In vitro translation products of total mRNA isolated from cells grown on 3% glucose (repression) or 1% methanol (induction) were analyzed on SDS-PAGE gels.

Figure 1 shows the comparison of in vitro translation products of mRNA from induced (lane 1), not induced (lane 2) cells, as well as the immunoprecipitates of the first preparation with specific antibodies directed against FMDH (lane 4). In addition, the electrophoretic patterns of crude protein extracts from 1% methanol, 0.5% glycerol/0.1% glucose (derepression) and 3% glucose cultures were compared with the electrophoretic mobility of purified FMDH (lanes 7, 8, 9 and 10 respectively).

The results obtained clearly identified the FMDH protein position on SDS-PAGE, and indicate that FMDH protein and its mRNA are predominant species in cells grown on methanol (induction). The position of two other predominant proteins, MOX and DHAS, is also indicated. Fig. 1 also points out that considerable expression is achieved under derepressed conditions (lane 8) and that 3% glucose represses the enzymes of methanol utilisation system. Above conclusions enabled us to isolate through sucrose gradient centrifugation mRNA fraction enriched in mRNA encoding FMDH (lane 3) in order to use it for screening procedure.

Screening for FMDH gene

The *H. polymorpha* DNA bank in Charon 4 phage was screened by differential plaque hybridisation (Materials and Methods) with radioactive ^{32}P -labelled mRNA from induced, not induced cells and with ^{32}P -mRNA from a fraction enriched in FMDH mRNA (Fig. 1, lane 3). Additionally, replica filters were hybridized with ^{32}P -DNA probes from clones encoding MOX and DAS genes (3, 5). The latter was done to identify and eliminate the clones encoding the two other strongly inducible genes. Desired phages were selected and their DNA further characterized.

Characterisation of recombinant clones

The initial identification of a clone was achieved by hybrid selection technique, restriction mapping and establishing the size of the mRNA encoded by a given clone.

Hybrid selection

DNA from Charon4 recombinant clone JM was covalently bound to DPTE S-500 matrix, mRNA complementary to JM clone was selected and its in vitro translation products analyzed. Fig. 1 shows that the hybrid selected mRNA gives upon in vitro translation a major peptide product of the same electrophoretic mobility as FMDH peptide (lane 5). When peptides from lane 5 were precipitated with specific antibodies (lane 6), a major band of a size of FMDH and additional weak band are visible. In control experiment with not-induced mRNA not detectable mRNA of FMDH character was selected by this technique. The presence of additional weak bands visible in lane 5 and 6 are probably artefacts of the used hybrid selection technique.

These data strongly suggest that clone JM contains FMDH gene.

Restriction map and the size and direction of transcription

Restriction map of clone JM and its subclones is shown in Fig. 2. DNA fragments encompassing the gene were identified by hybridizing the Southern blots with ^{32}P -labelled induced mRNA.

8.5 kb EcoRI *H. polymorpha* DNA fragment from clone JM contains a gene. A further analysis allowed to subclone the gene and its presumptive regulatory regions on HindIII/EcoRI 4.1 kb fragments in pBR325.

S1 mapping

Non-radioactive HindIII/EcoRI 4.1 kb fragment from plasmid p3M1 was isolated and annealed with induced and not-induced mRNA. The size of DNA protected by its cognate mRNA against the action of nuclease S1 was analyzed by agarose electrophoresis followed by Southern blotting and hybridization with appropriate ^{32}P -DNA in order to visualize the fragment. Fig. 3, lane 1 shows that induced mRNA protects 1.2 kb long DNA fragment. This indicates that the gene codes for a protein of about 35-37,000 daltons. This value was found for the FMDH protein. Since in this MW region FMDH is the only strongly inducible protein, this result supports the identification of the gene.

3' end of the gene, transcription direction and the amount of FMDH transcript

Two fragments containing the gene, 1.0 kb BamHI/PstI and 1.4 kb HindIII/BamHI, were isolated and a 3' end label was introduced at BamHI site. Only the label on the right (Fig. 3, lane 3-4), 1.4 kb HindIII/BamHI fragment was protected by annealing with mRNA indicating the direction of transcription from left to right (arrow in Fig. 2). This size of the band (lane 4) indicates that the 3' end of the gene is located 850 bp to the right of the BamHI site. This type of experiment was also used to roughly establish the amount of FMDH

mRNA species in total polyA⁺ mRNA isolated from cells grown under different conditions. A known amount of ³²P-3' end labelled DNA containing part of the gene was hybridized with varying amounts of mRNA. At DNA excess conditions, the radioactivity present in a band protected against S1 by a given amount of mRNA is a measure of the quantity of FMDH mRNA in the preparation. The data indicate that FMDH mRNA contributes about 7% ± 1% and 3% to 4% of total polyA⁺ mRNA in preparation from induced and derepressed growth condition respectively. Fig. 3, lanes a, b, c, d, shows the comparison of intensity of the DNA band resulting from S1 experiments where 3 ug of DNA was hybridized with 10 ug of total polyA⁺ mRNA. It is also clearly visible that in mid-log phase of 3% glucose (repression) cultures, only negligible amounts of FMDH transcript is visible whereas the same culture at stationary phase shows already considerable amounts of transcript. this is a good example of derepression phenomenon - in stationary phase, glucose is exhausted.

5' end of the gene

1.0kb BamHI/PstI fragment with a single 5' end label at BamHI yielded upon S1 mapping the multiple bands ranging from 255-265 bp (Fig. 3, lane 5). The comparison of this value with sequence data indicated that transcription starts around position -12 from the first ATG. The lain band shows the start at "A" surrounded by pyrimidine track.

Nucleotide sequence

The nucleotide sequence of FMDH gene and encompassing region was determined by Sanger (10) and Maxam-Gilbert methods (11). The fragments to be sequenced were generated by deleting with Bal31 DNA containing the gene. Fig. 4 shows that all regions of the gene were sequenced several times in both directions. In case of doubts, M13 method data were corrected by data obtained by Maxam-Gilbert methods. The nucleotide sequence is presented in Fig. 5. The gene contains an open reading frame (ORF) of 1,020 nucleotides and code for a protein of 340 Da. The protein MW, calculated from these data, is 35,700 Da which agrees well with the values obtained by SDS-PAGE of purified protein. The gene was conclusively identified as FMDH by comparing the N-end of the gene as derived from DNA sequence with the data obtained by NH-end analysis of the purified protein.

5'-3' end regions

In the 5'-control regulatory region of eukaryotes, a consensus sequence -3A(9)XX1AUG4GX6py was reported to be required in efficiently transcribed and translated genes (12, 13). In FMDH gene, the rule is only partly followed where the sequence -3AUG + 1AUG + 4AX + 6A is present. The first ATG is proceeded by stop codons in all reading frames. The sequence CTATAAATA involved in eukaryotes in the initiation of transcription is found at position -40. Other features assumed to play a role in transcriptional control in yeast *S. cerevisiae* like CAACAA or CACACA (12) not present in FMDH.

In most of the yeasts studied until now, the gene 3' end region contains characteristic sequences which, according to some authors, play a role in proper termination of transcription and serve as polyadenylation signals (14, 15). Zared and Sherman (16), and Bennetzen and Hall (17) assumed that a sequence T-rich...TAG...TAGT(or TATGT)...AT...TTT or T...TAAATAA...A(or G)...T...A...AT play these roles. In FMDH gene, similarity to these consensus Sequences is rarely found. When looking for some potential signals, some repeating sequences were found. Sequences TTGGA and TAGG repeat twice. AAATATAA, similar to animal polyadenylation signal, is located 30 bp downstream from the end of ORF.

Example 1:

In order to be able to study the functional regions of FMDH 5' upstream region, a series of deletion of this region was isolated. First, to obtain the promoter without the structural gene, a pUC type plasmid containing the 1.4 kb Bam HI fragment was subjected to Bat31 exonuclease treatment after the plasmid was linearised at a proper point. At the beginning, attention was focused on the promoter fragment which had the deletion at the position -5 from the first ATG; the fragment is called "-5 promoter". Also "-9" deleted promoter was used in some experiments.

The "-5 promoter" was fused to the open reading frame of the bacterial β -lactamase gene (Bla). The gene was used in the laboratory as a very suitable model for studying the expression of foreign protein under the control of yeast promoters.

The signal sequence of the β -lactamase was not present in the construction obtained, thus enabling the measurement of enzyme activity in yeast protein extracts. The fused DNA fragment was cloned into the plasmid containing *H. polymorpha* autonomously replicating sequence (HARS1) (Fig. 6), and *S. cerevisiae* Ura3 gene which serves as a marker for *H. polymorpha* transformation. The amount of β -lactamase produced in *H. polymorpha* transformants was measured by the enzymatic and immuno-tests. Table 1 shows the synthesis of β -lactamase under the control of FMDH promoter in cells grown in different media

(different carbon sources).

Table 1 shows that the isolated FMDH promoter is properly and stringently controlled by repression/derepression/induction mechanism. The estimation of the amount of synthesized protein shows that the system of this invention is characterised by very efficient transcription and translation of the foreign protein.

In the control experiment, β -lactamase was expressed in *S. cerevisiae* under the control of a strong *S. cerevisiae* PDC (pyruvate decarboxylase) promoter on 2- μ m plasmid (50 copies per cell). The values obtained were lower than in the case of *H. polymorpha* by a factor of 5-6.

TABLE 1

clone	Production of β -lactamase					
	enzymatic test (U/mg protein)			immuno-test (% of total cell protein)		
	GLU	GLYC	Met-OH	GLU	GLYC	Met-OH
Ir 45	30	4,000	15,000	-	3-4	6-8
L 5	70	10,000	28,000	-	6-8	10-12
GLU - growth on 3% glucose (repression)						
GLIC - growth on 1% glycerol (derepression)						
Met-OH - growth on 1% methanol (induction)						

In all cases, cells from late logarithmic phase were taken for measurement. The plasmid containing the fusion has 50-60 copies per cell.

Example 2:

Expression of genes encoding Hepatitis B surface antigens (HBSAg) under the control of FMDH promoter

1. Construction of the plasmid expressing the hepatitis proteins.

Hepatitis B 1,2 kb DNA fragment encodes a long S2-S1-S-protein (pre-s), which after processing (removal of S2-S1-part) is converted into the S-protein. Viral envelope consists of both proteins.

For our expression experiments we have used the 1,2 kb fragment as well as a shorter part of this DNA which encodes only S-protein. The latter is also able to form antigenic pseudo viral particles.

We have inserted both hepatitis S-gene into our universal vector. As shown in Fig. 1 and scheme 1 the vector contains autonomous replication sequence (HARS), URA3 gene from *S. cerevisiae* as a selective marker and *H. polymorpha* promoter followed by short linker. After the S-gene we have placed DNA fragment derived from *H. polymorpha* MOX gene exhibiting the transcription terminator function. Fig. 7 shows the construction containing the S-gene.

2. Transformation of *H. polymorpha* and screening for clones expressing HBSAg.

H. polymorpha URA3 mutant LR9 was transformed with the above described plasmids. The yeast transformants were then immediately screen for the expression of HBSAg using polyclonal antibodies. As an immuno-screening we have used Western blotting (peroxidase-protein A or to improve sensitivity 125 J - protein A). The screening procedure was considerably impeded by the strong cross-reactivity of the sera with *H. polymorpha* crude extract proteins. We were, however, able to show the expressed antigen.

Fig. 8 shows the Western blotting Protein extracts from cells transformed with hepatitis gene grown on methanol and shows an additional antigenic band having the expected MW of S-protein. The control extracts from transformants grown on glucose (repression of FMDH promoter) do not have this band. The results shown in Fig. 8 are coming from transformants containing FMDH -9 promoter i.e. promoter derived by deleting the DNA fragment encompassing the promoter function till position -9 from the first ATG.

We analysed also by testing S1-nuclease mapping mRNA produced in our transformants. The results indicate that transformants are producing a lot of S-gene mRNA species and that the transcription is stringently controlled by repression/derepression/induction mechanism.

The above results were confirmed by positive RIA TEST of protein extracts derived from transformed cells. In the test the monoclonal antibodies directed against native S-protein were used.

Example 3:

Expression and secretion of α -amylase from *Schwanniomyces castelli* in *H. polymorpha* under the control of FMDH promoter.

To study the possibility of expressing in *H. polymorpha* a secretory protein we have chosen α -amylase gene from yeast *S. castellii*. The gene encodes the 56 kd protein which in *S. castellii* is totally secreted into the medium; this secretory process is accompanied by glycosilation of the protein.

We have inserted EcoRI fragment encompassing the structural gene and its terminator into our expression plasmid (Fig. 9).

H. polymorpha was transformed with this plasmid and the transformants were tested for the expression and secretion of α -amylase using a starch degradation test (halo formation on starch-iodine plates) or enzyme kinetic test kit (α -amylase Merkotest A).

The results clearly show that α -amylase is produced under control of FMDH promoter. Moreover, the protein is secreted into the medium. Fig. 10 shows that in mid-log phase about 90% of the protein is secreted into the medium. Starch-iodine plate test confirmed these results (Fig. 11).

The data also show that it is possible to get a high expression level under derepressed conditions. This feature of the system is especially very valuable and important for biotechnological applications, i.e. the synthesis of foreign proteins can begin without addition of methanol as inducer simply by exhausting glucose in the medium and/or by the addition of glycerol. A system that can be handled in such an easy way by simultaneously providing a very effective expression yielding amounts of proteins applicable in the biotechnological industry has not been provided earlier.

In separate studies it has been shown that the other *H. polymorpha* promoters like MOX and DAS do not respond so strongly to derepression signals. In the case of DAS promoter, the expression under derepressed condition is additionally decreased by post-transcriptional control.

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Claims

Claims for the following Contracting States : BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

1. A DNA fragment characterized in that it comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the Hansenula polymorpha genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment.
2. The DNA fragment according to claim 1, characterized in that the gene codes for a wild type FMDH protein.
3. The DNA fragment according to any one of claims 1 or 2, characterized in that it has been modified by recombinant DNA technologies, while retaining its promoter function.
4. The DNA fragment according to claim 1, characterized in that it comprises the 5' region of the nucleotide sequence shown in Figure 5.
5. The DNA fragment according to any one of claims 1 to 4, characterized in that it further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.
6. The DNA fragment according to claim 5, characterized in that the said foreign gene is selected from genes encoding:
 - (a) Hepatitis B Virus S1-S2-S antigen
 - (b) Hepatitis B Virus S-antigen
 - (c) alpha-amylase from Schwanniomyces castellii
 - (d) glucoamylase from Schwanniomyces castellii
 - (e) invertase from Saccharomyces cerevisiae.
7. The DNA fragment according to any one of claims 1 to 6, characterized in that it comprises a DNA sequence coding for a secretory signal.
8. The DNA fragment according to claim 7, characterized in that the secretory signal is selected from: Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, Schwanniomyces castellii α -amylase and glucoamylase signals, Saccharomyces cerevisiae α -factor and invertase signals.
9. The DNA fragment according to any one of claims 1 to 8, characterized in that it has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.
10. A recombinant vector, characterized in that it contains the DNA fragment according to any one of claims 1 to 9.
11. A microorganism characterized in that it comprises a vector according to claim 10.
12. The microorganism according to claim 11, characterized in that it is a methylotrophic yeast.

13. The microorganism according to claim 12, characterized in that the yeast is selected from the genera *Candida*, *Hansenula* or *Pichia*.
14. The microorganism according to any one of claims 11 to 13, characterized in that it has received the vector according to claim 10 by transformation.
15. The microorganism according to any one of claims 11 to 14, characterized in that the vector is integrated into the genome of the microorganism or maintained as an extrachromosomal DNA molecule.
16. The microorganism according to any one of claims 11 to 15, characterized in that it tolerates high concentrations of foreign protein.
17. A process for producing a useful substance, characterized in that a microorganism according to any one of claims 11 to 16 is cultured and the useful substance is recovered and purified in a manner known in the art.

Claims for the following Contracting State : AT

1. A DNA fragment characterized in that it comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the *Hansenula polymorpha* genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment.
2. The DNA fragment according to claim 1, characterized in that the gene codes for a wild type FMDH protein.
3. The DNA fragment according to any one of claims 1 or 2, characterized in that it has been modified by recombinant DNA technologies, while retaining its promoter function.
4. The DNA fragment according to claim 1, characterized in that it comprises the 5' region of the nucleotide sequence shown in Figure 5.
5. The DNA fragment according to any one of claims 1 to 4, characterized in that it further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.
6. The DNA fragment according to claim 5, characterized in that the said foreign gene is selected from genes encoding:
 - (a) Hepatitis B Virus S1-S2-S antigen
 - (b) Hepatitis B Virus S-antigen
 - (c) alpha-amylase from *Schwanniomyces castellii*
 - (d) glucoamylase from *Schwanniomyces castellii*
 - (e) invertase from *Saccharomyces cerevisiae*.
7. The DNA fragment according to any one of claims 1 to 6, characterized in that it comprises a DNA sequence coding for a secretory signal.
8. The DNA fragment according to claim 7, characterized in that the secretory signal is selected from: *Hansenula polymorpha* membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, *Schwanniomyces castellii* α -amylase and glucoamylase signals, *Saccharomyces cerevisiae* α -factor and invertase signals.
9. The DNA fragment according to any one of claims 1 to 8, characterized in that it has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

10. A recombinant vector, characterized in that it contains the DNA fragment according to any one of claims 1 to 9.
11. A microorganism characterized in that it comprises a vector according to claim 10.
12. The microorganism according to claim 11, characterized in that it is a methylotrophic yeast.
13. The microorganism according to claim 12, characterized in that the yeast is selected from the genera *Candida*, *Hansenula* or *Pichia*.
14. The microorganism according to any one of claims 11 to 13, characterized in that it has received the vector according to claim 10 by transformation.
15. The microorganism according to any one of claims 11 to 14, characterized in that the vector is integrated into the genome of the microorganism or maintained as an extrachromosomal DNA molecule.
16. The microorganism according to any one of claims 11 to 15, characterized in that it tolerates high concentrations of foreign protein.
17. A process for producing a useful substance, characterized in that a microorganism according to any one of claims 11 to 16 is cultured and the useful substance is recovered and purified in a manner known in the art.
18. A process for preparing a DNA fragment, wherein said DNA fragment comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the *Hansenula polymorpha* genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment, by isolating said DNA fragment in a manner known per se.
19. The process according to claim 18, characterized in that the gene codes for a wild type FMDH protein.
20. The process according to any one of claims 18 or 19, characterized in that the DNA fragment has been modified by recombinant DNA technologies, while retaining its promoter function.
21. The process according to claim 18, characterized in that the DNA fragment comprises the 5' region of the nucleotide sequence shown in Figure 5.
22. The process according to any one of claims 18 to 21, characterized in that the DNA fragment further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.
23. The process according to claim 22, characterized in that the said foreign gene is selected from genes encoding:
 - (a) Hepatitis B Virus S1-S2-S antigen
 - (b) Hepatitis B Virus S-antigen
 - (c) alpha-amylase from *Schwanniomyces castellii*
 - (d) glucoamylase from *Schwanniomyces castellii*
 - (e) invertase from *Saccharomyces cerevisiae*.
24. The process according to any one of claims 18 to 23, characterized in that the DNA fragment comprises a DNA sequence coding for a secretory signal.
25. The process according to claim 24, characterized in that the secretory signal is selected from: *Hansenula polymorpha* membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, *Schwanniomyces castellii* α -amylase and glucoamylase signals, *Saccharomyces cerevisiae* α -factor and invertase signals.

26. The process according to any one of claims 18 to 25, characterized in that the DNA fragment has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.
- 5 27. A process for preparing a recombinant vector, characterized in that the DNA fragment produced according to any one of claims 18 to 26 is inserted into a cloning vector.
28. A process for preparing microorganism characterized in that a vector according to claim 27 is introduced into said microorganism.
- 10 29. The process according to claim 28, characterized in that the microorganism is a methylotrophic yeast.
30. The process according to claim 29, characterized in that the yeast is selected from the genera *Candida*, *Hansenula* or *Pichia*.
- 15 31. The process according to any one of claims 28 to 30, characterized in that the vector produced according to claim 27 has been introduced by transformation.
32. The process according to any one of claims 28 to 31, characterized in that the vector is integrated into
20 the genome of the microorganism or maintained as an extrachromosomal DNA molecule.
33. The process according to any one of claims 28 to 32, characterized in that the microorganism tolerates high concentrations of foreign protein.
- 25 34. A process for producing a useful substance, characterized in that a microorganism produced according to any one of claims 18 to 33 is cultured and the useful substance is recovered and purified in a manner known in the art.

Claims for the following Contracting State : ES

- 30 1. A process for preparing a DNA fragment, wherein said DNA fragment comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the *Hansenula polymorpha* genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII
35 fragment, by isolating said DNA fragment in a manner known per se.
2. The process according to claim 1, characterized in that the gene codes for a wild type FMDH protein.
3. The process according to any one of claims 1 or 2, characterized in that the DNA fragment has been
40 modified by recombinant DNA technologies, while retaining its promoter function.
4. The process according to claim 1, characterized in that the DNA fragment comprises the 5' region of the nucleotide sequence shown in Figure 5.
- 45 5. The process according to any one of claims 1 to 4, characterized in that the DNA fragment further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.
6. The process according to claim 5, characterized in that the said foreign gene is selected from genes
50 encoding:
 - (a) Hepatitis B Virus S1-S2-S antigen
 - (b) Hepatitis B Virus S-antigen
 - (c) alpha-amylase from *Schwanniomyces castellii*
 - (d) glucoamylase from *Schwanniomyces castellii*
 - 55 (e) invertase from *Saccharomyces cerevisiae*.
7. The process according to any one of claims 1 to 6, characterized in that the DNA fragment comprises a DNA sequence coding for a secretory signal.

8. The process according to claim 7, characterized in that the secretory signal is selected from:
Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins
methanol oxidase and dihydroxyacetone synthase, Schwanniomyces castellii α -amylase and
glucoamylase signals, Saccharomyces cerevisiae α -factor and invertase signals.
9. The process according to any one of claims 1 to 8, characterized in that the DNA fragment has been
obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter
region has been obtained from natural DNA and/or chemically synthesized DNA.
10. A process for preparing a recombinant vector, characterized in that the DNA fragment produced
according to any one of claims 1 to 9 is inserted into a cloning vector.
11. A process for preparing microorganism characterized in that a vector according to claim 10 is
introduced into said microorganism.
12. The process according to claim 11, characterized in that the microorganism is a methylotrophic yeast.
13. The process according to claim 12, characterized in that the yeast is selected from the genera Candida,
Hansenula or Pichia.
14. The process according to any one of claims 11 to 13, characterized in that the vector produced
according to claim 10 has been introduced by transformation.
15. The process according to any one of claims 11 to 14, characterized in that the vector is integrated into
the genome of the microorganism or maintained as an extrachromosomal DNA molecule.
16. The process according to any one of claims 11 to 15, characterized in that the microorganism tolerates
high concentrations of foreign protein.
17. A process for producing a useful substance, characterized in that a microorganism produced according
to any one of claims 11 to 16 is cultured and the useful substance is recovered and purified in a
manner known in the art.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. DNA-Fragment, dadurch gekennzeichnet, daß es einen Promoterbereich eines für ein Protein mit
Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen
Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem
Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-
Fragment angeordnet ist.
2. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein
kodiert.
3. DNA-Fragment nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß es durch rekombi-
nante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.
4. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß es den 5'-Bereich der in Figur 5
gezeigten Nukleotidsequenz umfaßt.
5. DNA-Fragment nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß es weiter mindestens
eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbe-
reiches umfaßt.
6. DNA-Fragment nach Anspruch 5, dadurch gekennzeichnet, daß das fremde Gen ausgewählt ist aus
Genen, die kodieren für:
(a) Hepatitis B Virus S1-S2-S Antigen

- (b) Hepatitis B Virus S-Antigen
- (c) alpha-Amylase aus Schwanniomyces castellii
- (d) Glucoamylase aus Schwanniomyces castellii
- (e) Invertase aus Saccharomyces cerevisiae.

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7. DNA-Fragment nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß es eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.

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8. DNA-Fragment nach Anspruch 7, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus:

Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroacetonsynthase, *Schwanniomyces castellii* α -Amylase- und Glucoamylasesignalen, *Saccharomyces cerevisiae* α -Faktor- und Invertasesignalen.

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9. DNA-Fragment nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß es aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten ist.

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10. Rekombinanter Vektor, dadurch gekennzeichnet, daß er das DNA-Fragment nach einem der Ansprüche 1 bis 9 enthält.

11. Mikroorganismus, dadurch gekennzeichnet, daß er einen Vektor nach Anspruch 10 umfaßt.

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12. Mikroorganismus nach Anspruch 11, dadurch gekennzeichnet, daß er eine methylotrophe Hefe ist.

13. Mikroorganismus nach Anspruch 12, dadurch gekennzeichnet, daß die Hefe aus den Gattungen *Candida*, *Hansenula* oder *Pichia* ausgewählt ist.

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14. Mikroorganismus nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß er den Vektor nach Anspruch 10 durch Transformation erhalten hat.

15. Mikroorganismus nach einem der Ansprüche 11 bis 14, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert ist oder als extrachromosomales DNA-Molekül erhalten bleibt.

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16. Mikroorganismus nach einem der Ansprüche 11 bis 15, dadurch gekennzeichnet, daß er hohe Konzentrationen von fremdem Protein toleriert.

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17. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein Mikroorganismus nach einem der Ansprüche 11 bis 16 kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

Patentansprüche für folgenden Vertragsstaat : AT

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1. DNA-Fragment, dadurch gekennzeichnet, daß es einen Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem *Hansenula polymorpha* Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist.

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2. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein kodiert.

3. DNA-Fragment nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß es durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.

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4. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß es den 5'-Bereich der in Figur 5 gezeigten Nukleotidsequenz umfaßt.

5. DNA-Fragment nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß es weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbereiches umfaßt.
- 5 6. DNA-Fragment nach Anspruch 5, dadurch gekennzeichnet, daß das fremde Gen ausgewählt ist aus Genen, die kodieren für:
 - (a) Hepatitis B Virus S1-S2-S Antigen
 - (b) Hepatitis B Virus S-Antigen
 - (c) alpha-Amylase aus Schwanniomyces castellii
 - 10 (d) Glucoamylase aus Schwanniomyces castellii
 - (e) Invertase aus Saccharomyces cerevisiae.
7. DNA-Fragment nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß es eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.
- 15 8. DNA-Fragment nach Anspruch 7, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus:

Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroaceton synthase, Schwanniomyces castellii α -Amylase- und

20 Glucoamylasesignalen, Saccharomyces cerevisiae α -Faktor- und Invertasesignalen.
9. DNA-Fragment nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß es aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten ist.
- 25 10. Rekombinanter Vektor, dadurch gekennzeichnet, daß er das DNA-Fragment nach einem der Ansprüche 1 bis 9 enthält.
11. Mikroorganismus, dadurch gekennzeichnet, daß er einen Vektor nach Anspruch 10 umfaßt.
- 30 12. Mikroorganismus nach Anspruch 11, dadurch gekennzeichnet, daß er eine methylotrophe Hefe ist.
13. Mikroorganismus nach Anspruch 12, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.
- 35 14. Mikroorganismus nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß er den Vektor nach Anspruch 10 durch Transformation erhalten hat.
15. Mikroorganismus nach einem der Ansprüche 11 bis 14, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert ist oder als extrachromosomales DNA-Molekül erhalten bleibt.
- 40 16. Mikroorganismus nach einem der Ansprüche 11 bis 15, dadurch gekennzeichnet, daß er hohe Konzentrationen von fremdem Protein toleriert.
- 45 17. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein Mikroorganismus nach einem der Ansprüche 11 bis 16 kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.
- 50 18. Verfahren zum Herstellen eines DNA-Fragmentes, wobei das DNA-Fragment den Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist, indem das DNA-Fragment auf übliche Weise isoliert wird.
- 55 19. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein kodiert.

20. Verfahren nach einem der Ansprüche 18 oder 19, dadurch gekennzeichnet, daß das DNA-Fragment durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.
- 5 21. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß das DNA-Fragment den 5'-Bereich der in Figur 5 gezeigten Nukleotidssequenz umfaßt.
22. Verfahren nach einem der Ansprüche 18 bis 21, dadurch gekennzeichnet, daß das DNA-Fragment weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbereiches umfaßt.
- 10 23. Verfahren nach Anspruch 22, dadurch gekennzeichnet, daß das fremde Gen aus den Genen ausgewählt ist, die kodieren für:
 - (a) Hepatitis B Virus S1-S2-S-Antigen
 - 15 (b) Hepatitis B Virus S-Antigen
 - (c) alpha-Amylase aus Schwanniomyces castellii
 - (d) Glucoamylase aus Schwanniomyces castellii
 - (e) Invertase aus Saccharomyces cerevisiae.
- 20 24. Verfahren nach einem der Ansprüche 18 bis 23, dadurch gekennzeichnet, daß das DNA-Fragment eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.
- 25 25. Verfahren nach Anspruch 24, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus: Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroxyacetonsynthase, Schwanniomyces castellii α -Amylase- und Glucoamylasesignalen, Saccharomyces cerevisiae α -Faktor- und Invertasesignalen.
- 30 26. Verfahren nach einem der Ansprüche 18 bis 25, dadurch gekennzeichnet, daß das DNA-Fragment aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten worden ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten worden ist.
27. Verfahren zum Herstellen eines rekombinanten Vektors, dadurch gekennzeichnet, daß das nach einem der Ansprüche 18 bis 26 erzeugte DNA-Fragment in einen Klonierungsvektor inseriert wird.
- 35 28. Verfahren zum Herstellen eines Mikroorganismus, dadurch gekennzeichnet, daß ein Vektor nach Anspruch 27 in den Mikroorganismus eingeführt wird.
29. Verfahren nach Anspruch 28, dadurch gekennzeichnet, daß der Mikroorganismus eine methylotrophe Hefe ist.
- 40 30. Verfahren nach Anspruch 29, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.
31. Verfahren nach einem der Ansprüche 28 bis 30, dadurch gekennzeichnet, daß der nach Anspruch 27 erzeugte Vektor durch Transformation eingeführt wird.
- 45 32. Verfahren nach einem der Ansprüche 28 bis 31, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert wird oder als ein extrachromosomales DNA-Molekül erhalten bleibt.
- 50 33. Verfahren nach einem der Ansprüche 28 bis 32, dadurch gekennzeichnet, daß der Mikroorganismus hohe Konzentrationen von fremdem Protein toleriert.
- 55 34. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein nach einem der Ansprüche 18 bis 33 erzeugter Mikroorganismus kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zum Herstellen eines DNA-Fragmentes, wobei das DNA-Fragment den Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von
5 einer methylophilen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist, indem das DNA-Fragment auf übliche Weise isoliert wird.
2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein
10 kodiert.
3. Verfahren nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß das DNA-Fragment durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.
- 15 4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das DNA-Fragment den 5'-Bereich der in Figur 5 gezeigten Nukleotidssequenz umfaßt.
5. Verfahren nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß das DNA-Fragment weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des
20 Promoterbereiches umfaßt.
6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß das fremde Gen aus den Genen ausgewählt ist, die kodieren für:
25 (a) Hepatitis B Virus S1-S2-S-Antigen
(b) Hepatitis B Virus S-Antigen
(c) alpha-Amylase aus Schwanniomyces castellii
(d) Glucoamylase aus Schwanniomyces castellii
(e) Invertase aus Saccharomyces cerevisiae.
- 30 7. Verfahren nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß das DNA-Fragment eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.
8. Verfahren nach Anspruch 7, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus:
35 Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroxyacetonsynthase, Schwanniomyces castellii α -Amylase- und Glucoamylasesignalen, Saccharomyces cerevisiae α -Faktor- und Invertasesignalen.
9. Verfahren nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß das DNA-Fragment aus
40 natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten worden ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten worden ist.
10. Verfahren zum Herstellen eines rekombinanten Vektors, dadurch gekennzeichnet, daß das nach einem der Ansprüche 1 bis 9 erzeugte DNA-Fragment in einen Klonierungsvektor inseriert wird.
- 45 11. Verfahren zum Herstellen eines Mikroorganismus, dadurch gekennzeichnet, daß ein Vektor nach Anspruch 10 in den Mikroorganismus eingeführt wird.
12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, daß der Mikroorganismus eine methylophile
50 Hefe ist.
13. Verfahren nach Anspruch 12, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.
14. Verfahren nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß der nach Anspruch 10
55 erzeugte Vektor durch Transformation eingeführt wird.
15. Verfahren nach einem der Ansprüche 11 bis 14, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert wird oder als ein extrachromosomales DNA-Molekül erhalten

bleibt.

16. Verfahren nach einem der Ansprüche 11 bis 15, dadurch gekennzeichnet, daß der Mikroorganismus hohe Konzentrationen von fremdem Protein toleriert.

5

17. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein nach einem der Ansprüche 11 bis 16 erzeugter Mikroorganismus kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

10 **Revendications**

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Fragment d'ADN caractérisé en ce qu'il comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment *Bam*HI-*Hind*III de 3,5 kb.

15

2. Fragment d'ADN selon la revendication 1, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.

20

3. Fragment d'ADN selon l'une quelconque des revendications 1 ou 2, caractérisé en ce qu'il est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.

4. Fragment d'ADN selon la revendication 1, caractérisé en ce qu'il comprend la région 5' de la séquence de nucléotides présentée à la figure 5.

25

5. Fragment d'ADN selon l'une quelconque des revendications 1 à 4, caractérisé en ce qu'il comprend en outre au moins une séquence d'ADN codant un gène étranger sous le contrôle de transcription de la région promotrice.

30

6. Fragment d'ADN selon la revendication 5, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant :

- (a) l'antigène S1-S2-S du virus de l'hépatite B
- (b) l'antigène S du virus de l'hépatite B
- (c) une alpha-amylase de *Schwanniomyces castellii*
- (d) une glucoamylase de *Schwanniomyces castellii*
- (e) une invertase de *Saccharomyces cerevisiae*.

35

7. Fragment d'ADN selon l'une quelconque des revendications 1 à 6, caractérisé en ce qu'il comprend une séquence d'ADN codant pour un signal sécrétoire.

40

8. Fragment d'ADN selon la revendication 7, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des alpha-amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des alpha-facteur et invertase de *Saccharomyces cerevisiae*.

45

9. Fragment d'ADN selon l'une quelconque des revendications 1 à 8, caractérisé en ce qu'il est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

50

10. Vecteur recombinant, caractérisé en ce qu'il contient le fragment d'ADN selon l'une quelconque des revendications 1 à 9.

55

11. Micro-organisme caractérisé en ce qu'il comprend un vecteur selon la revendication 10.

12. Micro-organisme selon la revendication 11, caractérisé en ce que c'est une levure méthylotrophe.

13. Micro-organisme selon la revendication 12, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.

14. Micro-organisme selon l'une quelconque des revendications 11 à 13, caractérisé en ce qu'il reçoit le vecteur selon la revendication 10 par transformation.

15. Micro-organisme selon l'une quelconque des revendications 11 à 14, caractérisé en ce que le vecteur est intégré dans le génome du microorganisme ou maintenu comme molécule d'ADN extrachromosomique.

16. Micro-organisme selon l'une quelconque des revendications 11 à 15, caractérisé en ce qu'il tolère des concentrations élevées de protéine étrangère.

17. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme selon l'une quelconque des revendications 11 à 16 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

Revendications pour l'Etat contractant suivant : AT

1. Fragment d'ADN caractérisé en ce qu'il comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment BamHI-HindIII de 3,5 kb.

2. Fragment d'ADN selon la revendication 1, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.

3. Fragment d'ADN selon l'une quelconque des revendications 1 ou 2, caractérisé en ce qu'il est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.

4. Fragment d'ADN selon la revendication 1, caractérisé en ce qu'il comprend la région 5' de la séquence de nucléotides présentée à la figure 5.

5. Fragment d'ADN selon l'une quelconque des revendications 1 à 4, caractérisé en ce qu'il comprend en outre au moins une séquence d'ADN codant pour un gène étranger sous le contrôle de transcription de la région promotrice.

6. Fragment d'ADN selon la revendication 5, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant pour :

- (a) l'antigène S1-S2-S du virus de l'hépatite B
- (b) l'antigène S du virus de l'hépatite B
- (c) l'alpha-amylase de *Schwanniomyces castellii*
- (d) la glucoamylase de *Schwanniomyces castellii*
- (e) l'invertase de *Saccharomyces cerevisiae*.

7. Fragment d'ADN selon l'une quelconque des revendications 1 à 6, caractérisé en ce qu'il comprend une séquence d'ADN codant pour un signal sécrétoire.

8. Fragment d'ADN selon la revendication 7, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de la membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des α -amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des α -facteur et invertase de *Saccharomyces cerevisiae*.

9. Fragment d'ADN selon l'une quelconque des revendications 1 à 8, caractérisé en ce qu'il est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

10. Vecteur recombinant, caractérisé en ce qu'il contient le fragment d'ADN selon l'une quelconque des revendications 1 à 9.
11. Micro-organisme caractérisé en ce qu'il comprend un vecteur selon la revendication 10.
12. Microorganisme selon la revendication 11, caractérisé en ce que c'est une levure méthylotrophe.
13. Micro-organisme selon la revendication 12, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.
14. Micro-organisme selon l'une quelconque des revendications 11 à 13, caractérisé en ce qu'il reçoit le vecteur selon la revendication 10 par transformation.
15. Micro-organisme selon l'une quelconque des revendications 11 à 14, caractérisé en ce que le vecteur est intégré dans le génome du microorganisme ou maintenu comme molécule d'ADN extrachromosomique.
16. Micro-organisme selon l'une quelconque des revendications 11 à 15, caractérisé en ce qu'il tolère des concentrations élevées de protéine étrangère.
17. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme selon l'une quelconque des revendications 11 à 16 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.
18. Procédé pour préparer un fragment d'ADN, dans lequel ledit fragment d'ADN comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment BamHI-HindIII de 3,5 kb, en isolant ledit fragment d'ADN d'une manière connue en soi.
19. Procédé selon la revendication 18, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.
20. Procédé selon l'une quelconque des revendications 18 ou 19, caractérisé en ce que le fragment d'ADN est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.
21. Procédé selon la revendication 18, caractérisé en ce que le fragment d'ADN comprend la région 5' de la séquence de nucléotides présentée à la figure 5.
22. Procédé selon l'une quelconque des revendications 18 à 21, caractérisé en ce que le fragment d'ADN comprend en outre au moins une séquence d'ADN codant pour un gène étranger sous le contrôle de transcription de la région promotrice.
23. Procédé selon la revendication 22, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant pour :
 - (a) l'antigène S1-S2-S du virus de l'hépatite B
 - (b) l'antigène S du virus de l'hépatite B
 - (c) l'alpha-amylase de *Schwanniomyces castellii*
 - (d) la glucoamylase de *Schwanniomyces castellii*
 - (e) l'invertase de *Saccharomyces cerevisiae*.
24. Procédé selon l'une quelconque des revendications 18 à 23, caractérisé en ce que le fragment d'ADN comprend une séquence d'ADN codant pour un signal sécrétoire.
25. Procédé selon la revendication 24, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de la membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des

α -amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des α -facteur et invertase de *Saccharomyces cerevisiae*.

26. Procédé selon l'une quelconque des revendications 18 à 25, caractérisé en ce que le fragment d'ADN est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.
27. Procédé pour préparer un vecteur recombinant, caractérisé en ce que le fragment d'ADN obtenu selon l'une quelconque des revendications 18 à 26 est inséré dans un vecteur clonant.
28. Procédé pour préparer un micro-organisme caractérisé en ce qu'un vecteur selon la revendication 27 est introduit dans ledit micro-organisme.
29. Procédé selon la revendication 28, caractérisé en ce que le micro-organisme est une levure méthylotrophe.
30. Procédé selon la revendication 29, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.
31. Procédé selon l'une quelconque des revendications 28 à 30, caractérisé en ce que le vecteur obtenu selon la revendication 27 est introduit par transformation.
32. Procédé selon l'une quelconque des revendications 28 à 31, caractérisé en ce que le vecteur est intégré dans le génome du micro-organisme ou maintenu comme molécule d'ADN extrachromosomique.
33. Procédé selon l'une quelconque des revendications 28 à 32, caractérisé en ce que le micro-organisme tolère des concentrations élevées de protéine étrangère.
34. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme obtenu selon l'une quelconque des revendications 18 à 33 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

Revendications pour l'Etat contractant suivant : ES

1. Procédé pour préparer un fragment d'ADN, dans lequel ledit fragment d'ADN comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment BamHI-HindIII de 3,5 kb, en isolant ledit fragment d'ADN d'une manière connue en soi.
2. Procédé selon la revendication 1, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.
3. Procédé selon l'une quelconque des revendications 1 ou 2, caractérisé en ce que le fragment d'ADN est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.
4. Procédé selon la revendication 1, caractérisé en ce que le fragment d'ADN comprend la région 5' de la séquence de nucléotides présentée à la figure 5.
5. Procédé selon l'une quelconque des revendications 1 à 4, caractérisé en ce que le fragment d'ADN comprend en outre au moins une séquence d'ADN codant pour un gène étranger sous le contrôle de transcription de la région promotrice.
6. Procédé selon la revendication 5, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant pour :
 - (a) l'antigène S1-S2-S du virus de l'hépatite B

- (b) l'antigène S du virus de l'hépatite B.
- (c) l'alpha-amylase de *Schwanniomyces castellii*
- (d) la glucoamylase de *Schwanniomyces castellii*
- (e) l'invertase de *Saccharomyces cerevisiae*.

5

7. Procédé selon l'une quelconque des revendications 1 à 6, caractérisé en ce que le fragment d'ADN comprend une séquence d'ADN codant pour un signal sécrétoire.

10 8. Procédé selon la revendication 7, caractérisé en ce que le signal sécrétoire est choisi parmi :
les signaux de translocation de la membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des α -amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des α -facteur et invertase de *Saccharomyces cerevisiae*.

15 9. Procédé selon l'une quelconque des revendications 1 à 8, caractérisé en ce que le fragment d'ADN est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

20 10. Procédé pour préparer un vecteur recombinant, caractérisé en ce que le fragment d'ADN obtenu selon l'une quelconque des revendications 1 à 9 est inséré dans un vecteur clonant.

11. Procédé pour préparer un micro-organisme caractérisé en ce qu'un vecteur selon la revendication 10 est introduit dans ledit micro-organisme.

25 12. Procédé selon la revendication 11, caractérisé en ce que le micro-organisme est une levure méthylotrophe.

13. Procédé selon la revendication 12, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.

30

14. Procédé selon l'une quelconque des revendications 11 à 13, caractérisé en ce que le vecteur obtenu selon la revendication 10 est introduit par transformation.

35 15. Procédé selon l'une quelconque des revendications 11 à 14, caractérisé en ce que le vecteur est intégré dans le génome du micro-organisme ou maintenu comme molécule d'ADN extrachromosomique.

16. Procédé selon l'une quelconque des revendications 11 à 15, caractérisé en ce que le micro-organisme tolère des concentrations élevées de protéine étrangère.

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17. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme obtenu selon l'une quelconque des revendications 11 à 16 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

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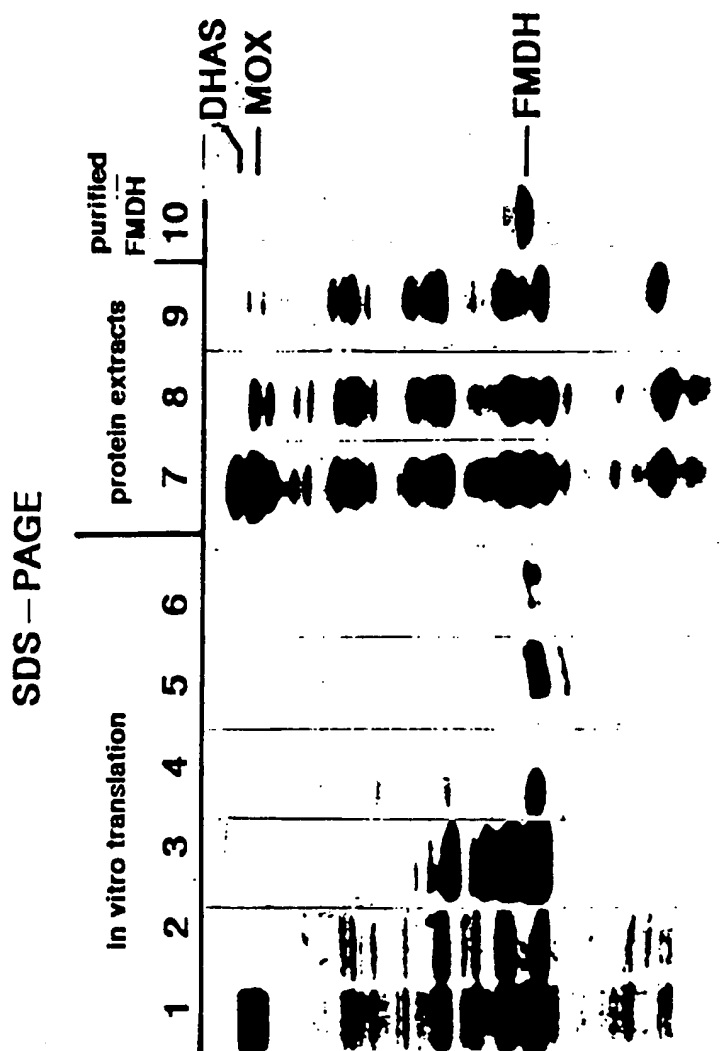


FIG.1. Analysis of protein crude extracts and in vitro translation products by SDS -polyacrylamid gel electrophoresis.

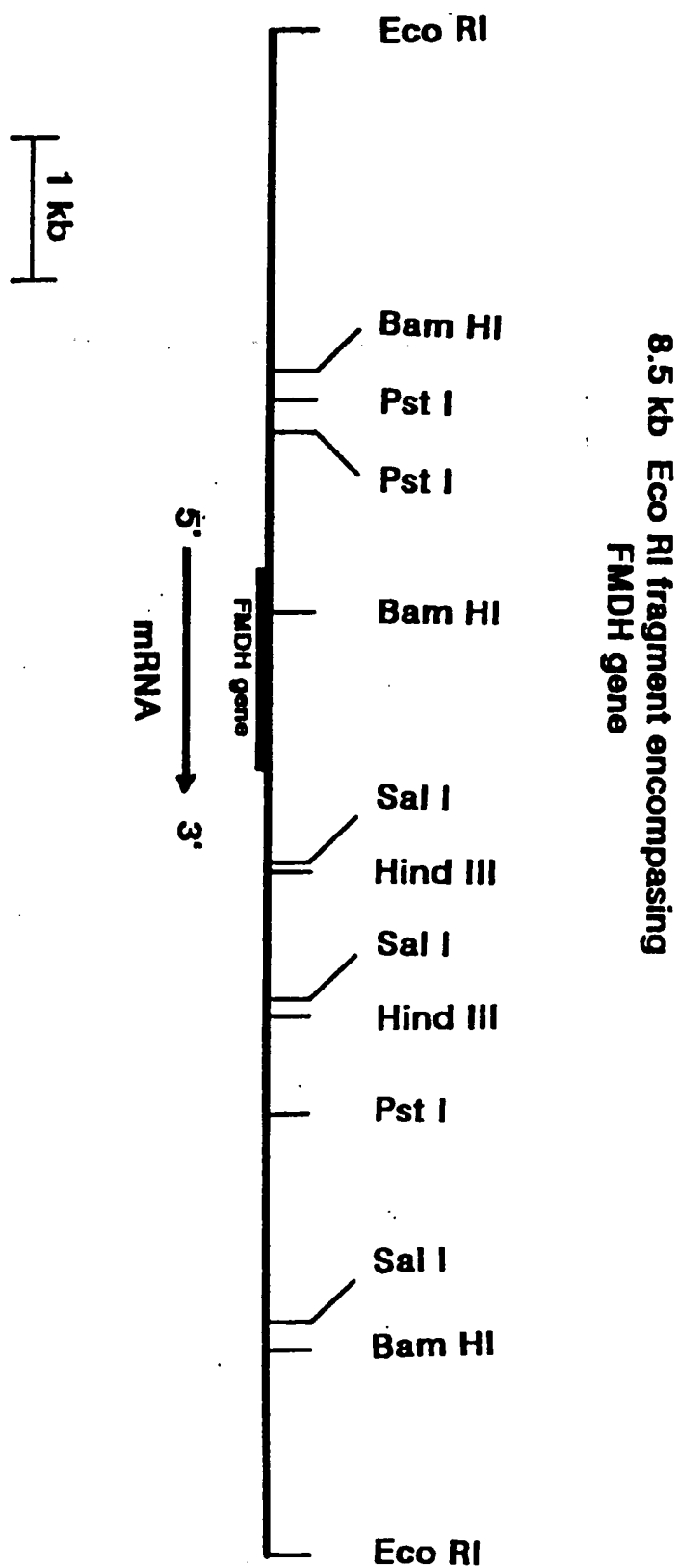


FIG.2. Restriction map of DNA fragment encompassing the FMDH gene.

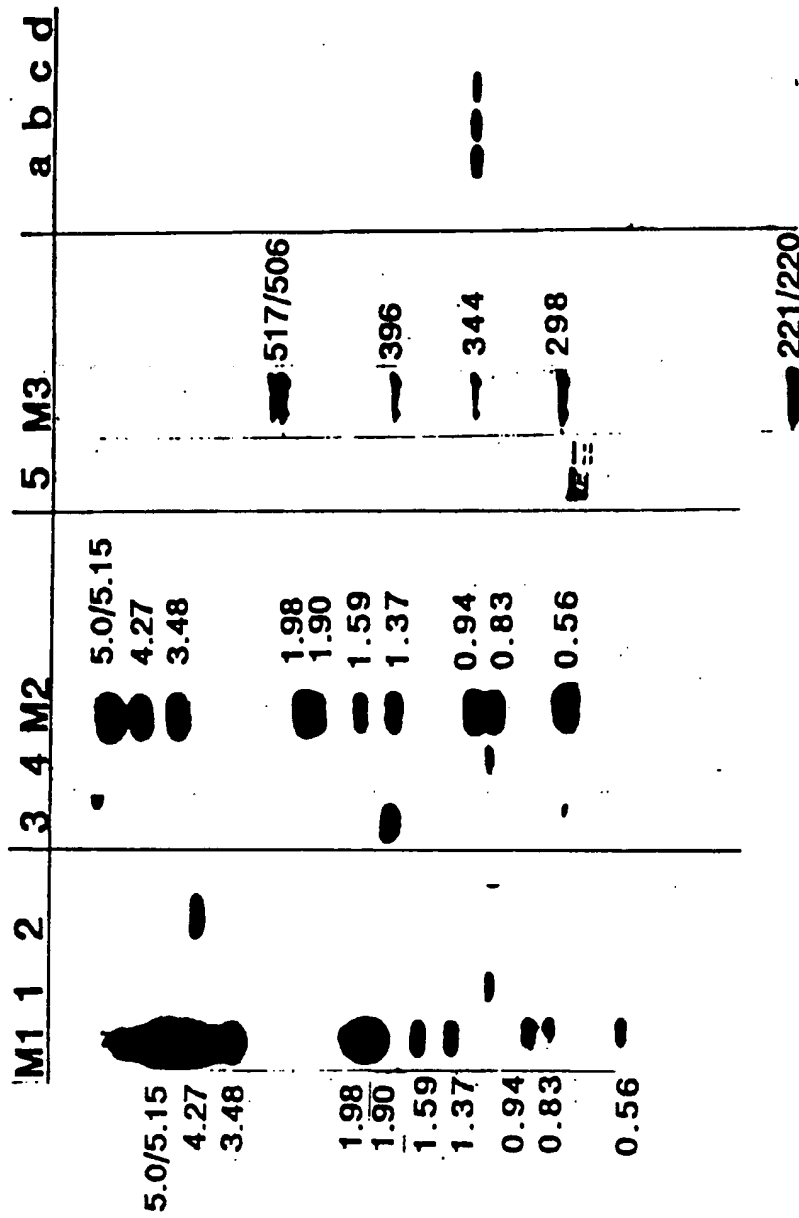


FIG. 3 S1-mapping

Sequencing strategy

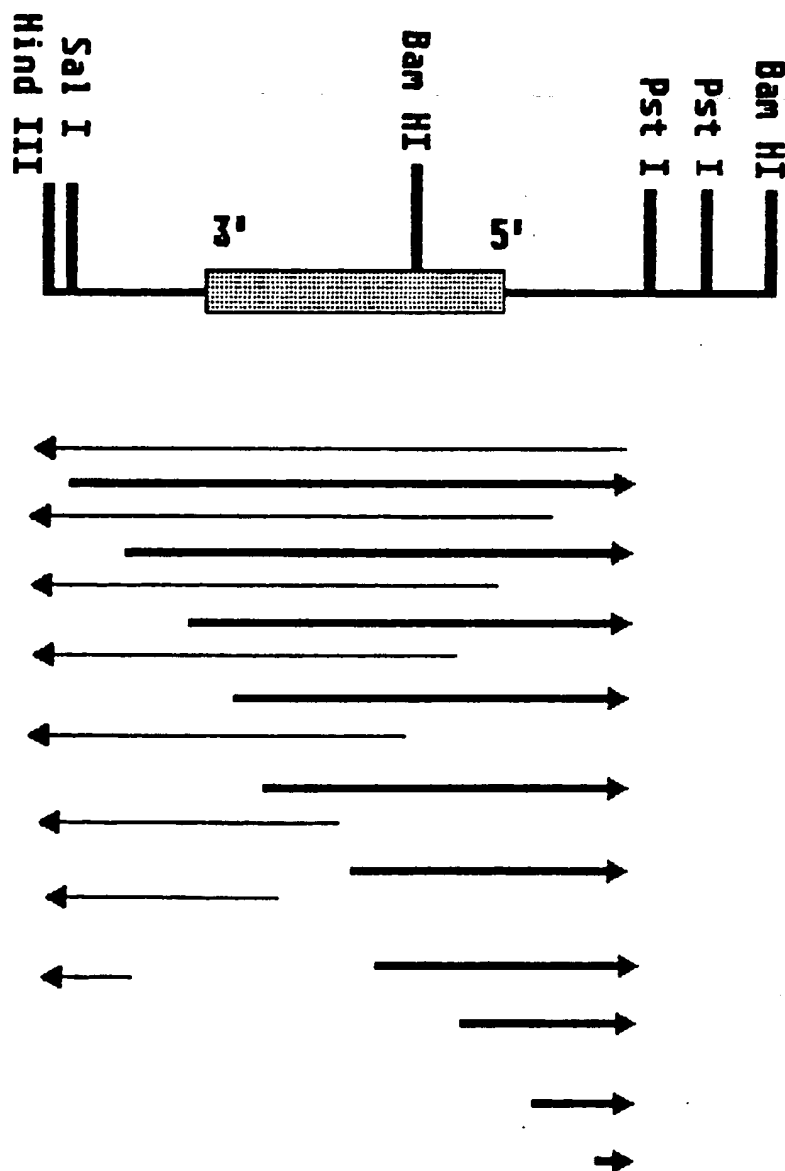


FIG.4 Sequencing strategy - schematic representation

FIG. 5a. Nucleotide sequence of FMDH gene and its 5',3' control regions.

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      10      20      30      40      50      60
ATCGCAGAAATGTATCTAAACGCAAACTCCGAGCTGGAAAAATGTTACCGGCGATGCGCG

      70      80      90     100     110     120
GACAATTTAGAGGCGGCAATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCT

     130     140     150     160     170     180
TCGATGGGCCCGAGATCCCACCGCGTTCCTGCGTACCGGGACGTGAGGCAGCGCGACATC

     190     200     210     220     230     240
CTACAAATATACCAGGCGCCAACCGAGTCTCTCGGAAAACACAGCTTCTGGATATCTTCC

     250     260     270     280     290     300
GCGGCGGGCGCAACGAGCCAAGAATAGTCCCTGGAGGTGACGGAATATATATGTGTGGAGG

     310     320     330     340     350     360
GTAAATCTGACAGGGTGTAGCAAAGTAATATTTTCTAAACATGCAATCGGCTGCCCC

     370     380     390     400     410     420
GCAACGGGAAAAAGAATGACTTTGGCACTCTTCACCAGAGTGGGGTGTCCCGCTCGTGTG

     430     440     450     460     470     480
TGCAATAGGCTCCCACTGGTCACCCCGGATTTTGAGAAAAACAGCAAGTTCGGGGTG

     490     500     510     520     530     540
TCTCACTGGTGTCCGCCAATAAGAGGACCGGCAGGCACGGAGTCTACATCAAGCTGTCTC

     550     560     570     580     590     600
CGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGCACTATAAATACCGCC

     610     620     630     640     650     660
TCCTTGGCTCTCTGCCTTCATCAATCAAATCATGAAGGTTGTACTAGTTCTCTACGACG
      MetLysValValLeuValLeuTyrAspA

     670     680     690     700     710     720
CAGGAAAACACGCCCAAGACGAGGAAAGACTCTACGGTTGCACTGAAAACGCCCTTGGA
1aGlyLysHisAlaGlnAspGluGluArgLeuTyrGlyCysThrGluAsnAlaLeuGlyI

     730     740     750     760     770     780
TCAGGGACTGGCTCGAGAAGCAGGGCCACGACGTCGTTGTCAACAGTGACAAGGAGGGGC
1aArgAspTrpLeuGluLysGlnGlyHisAspValValValThrSerAspLysGluGlyG

     790     800     810     820     830     840
AGAACTCTGTGCTCGAGAAGAACATCTCGGACGCAGATGTATCATCTCCACTCCTTTCC
1nAsnSerValLeuGluLysAsnIleSerAspAlaAspValIleIleSerThrProPheH

     850     860     870     880     890     900
ACCCAGCATACATACCAAGGAGAGAATCGACAAGGCCAAGAAGCTCAAGCTACTGGTGG
1aProAlaTyrIleThrLysGluArgIleAspLysAlaLysLysLeuLysLeuLeuValV

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910 920 930 940 950 960
 TTGCCGAGTGGGATCCGACCACATCGACCTTGACTACATCAACCAGTCCGGCAGAGACA
 alAlaGlyValGlySerAspHisIleAspLeuAspTyrIleAsnGlnSerGlyArgAspI

970 980 990 1000 1010 1020
 TTTCTGTGCTGGAGBTGACCGGTTGGAACGTCGTTTCGGTTGCCGAGCACGTTGTGATGA
 leSerValLeuGluValThrGlySerAsnValValSerValAlaGluHisValValMetT

1030 1040 1050 1060 1070 1080
 CGATGCTGGTGGTGGTGAGGAACCTTTGTTCCCTGCTCACGAGCAGATCATCTCTGGCGGCT
 hrMetLeuValLeuValArgAsnPheValProAlaHisGluGlnIleIleSerGlyGlyT

1090 1100 1110 1120 1130 1140
 GGAACGTGGCCGAGATCGCCAAGGACTCCTTCGACATCGAGGGCAAGGTCATTGCCACCA
 rpAsnValAlaGluIleAlaLysAspSerPheAspIleGluGlyLysValIleAlaThrI

1150 1160 1170 1180 1190 1200
 TCGGAGCAGGCAGAATCGGCTACCGTGTGCTGGAGAGACTTGTGGCCTTCAACCCTAAGG
 leGlyAlaGlyArgIleGlyTyrArgValLeuGluArgLeuValAlaPheAsnProLysG

1210 1220 1230 1240 1250 1260
 AGCTGCTCTACTACGACTACCAGTCGCTGTGCGAAGAGGCGGAGGAGAAAGTCGGCGCCC
 luLeuLeuTyrTyrAspTyrGlnSerLeuSerLysGluAlaGluGluLysValGlyAlaA

1270 1280 1290 1300 1310 1320
 GCAGAGTCCACGACATCAAGGAGCTGGTTGCCCGAGGCCGACATTGTCTACGATCAACTGTC
 rgArgValHisAspIleLysGluLeuValAlaGlnAlaAspIleValThrIleAsnCysP

1330 1340 1350 1360 1370 1380
 CACTGCACGCCGGCTCGAAGGGCCTGGTGAACGCAGAGCTGCTCAAGCACTTCAAGAAGG
 roLeuHisAlaGlySerLysGlyLeuValAsnAlaGluLeuLeuLysHisPheLysLysG

1390 1400 1410 1420 1430 1440
 GCGCCTGGCTCGTCAACACCGCCAGAGGTGCCATCTGCGTGGCCGAGGACGTTGCAGCCG
 lyAlaTrpLeuValAsnThrAlaArgGlyAlaIleCysValAlaGluAspValAlaAlaA

1450 1460 1470 1480 1490 1500
 CCGTCAAGAGCGGACAGCTTAGAGGATACGGTGGAGACGTGTGGTTCCACAGCCAGCTC
 laValLysSerGlyGlnLeuArgGlyTyrGlyGlyAspValTrpPheProGlnProAlaP

1510 1520 1530 1540 1550 1560
 CAAAGGACCACCCATGGAGATCCATGGCCAACAAGTACGGTGGCTGGCAATGCCATGACTC
 roLysAspHisProTrpArgSerMetAlaAsnLysTyrGlyAlaGlyAsnAlaMetThrP

1570 1580 1590 1600 1610 1620
 CGCACTACTCGGGCTCTGTCTATTGACGCCCAGGTCAGATACGCGCAGGGCACCAAGAACA
 roHisTyrSerGlySerValIleAspAlaGlnValArgTyrAlaGlnGlyThrLysAsnI

1630 1640 1650 1660 1670 1680
 TCCTGGAGTCGTTCTTCACTCAGAAGTTCGACTACAGGCCCCAGGACATCATTCTGCTGA
 leLeuGluSerPhePheThrGlnLysPheAspTyrArgProGlnAspIleIleLeuLeuA

1690 1700 1710 1720 1730 1740
 ACGGCAAGTACAAGACCAAGTCGTACGGTGCCGACAAATGAGCGGTCTTGAGGAGCTGA
 snGlyLysTyrLysThrLysSerTyrGlyAlaAspLysEnd

1750 1760 1770 1780 1790 1800
 TTGGATCTAGATGAAATAGGAAATATAATTATGGCTCTACTGCGCTGCGTAAACGTCACT

FIG.5 Nucleotide sequence of FMDH gene and its 5',3' control regions.

fmdh1

```

      1810      1820      1830      1840      1850      1860
GTAGGCGATTTCGCTTAGCCCAAGTCCGCGATGCGGTCCGACGACACCAGAGCGCGTCCA

      1870      1880      1890      1900      1910      1920
CCTCCTGTGCGCCGCACCGCCCCCAAGGAGGTTGCGGCTGTGCGGCTCGACGCGACCAA

      1930      1940      1950      1960      1970      1980
AAAAATAAGCGTCAAAAGGAGGTGTGAGGGAAGCACGCCGTGGGGCTCGAGATATATAAA

      1990      2000      2010      2020      2030      2040
GCGCAGCGTAGCTTTGTCTGTCTTGCTAATGAGCGACGACCAAGCCTTGGAATTTTCTCT

      2050      2060      2070      2080      2090      2100
GAAATCCCCCGTCACCCAGGACATGATCCACCACTTGGTGACGGTCACCTTACAGGTTCT

      2110      2120      2130      2140      2150      2160
GCCTTGCGAGTCTCTCAAGACCATCACCCAGAAAGTCAAGTCTTCAGCCGACACAGAGCC

      2170      2180      2190      2200      2210      2220
CGTGCTCAAGACCAAGCCGCTGCCCTCGCTGCATGACTTTCACCAAGCTCGTCCGCTATA

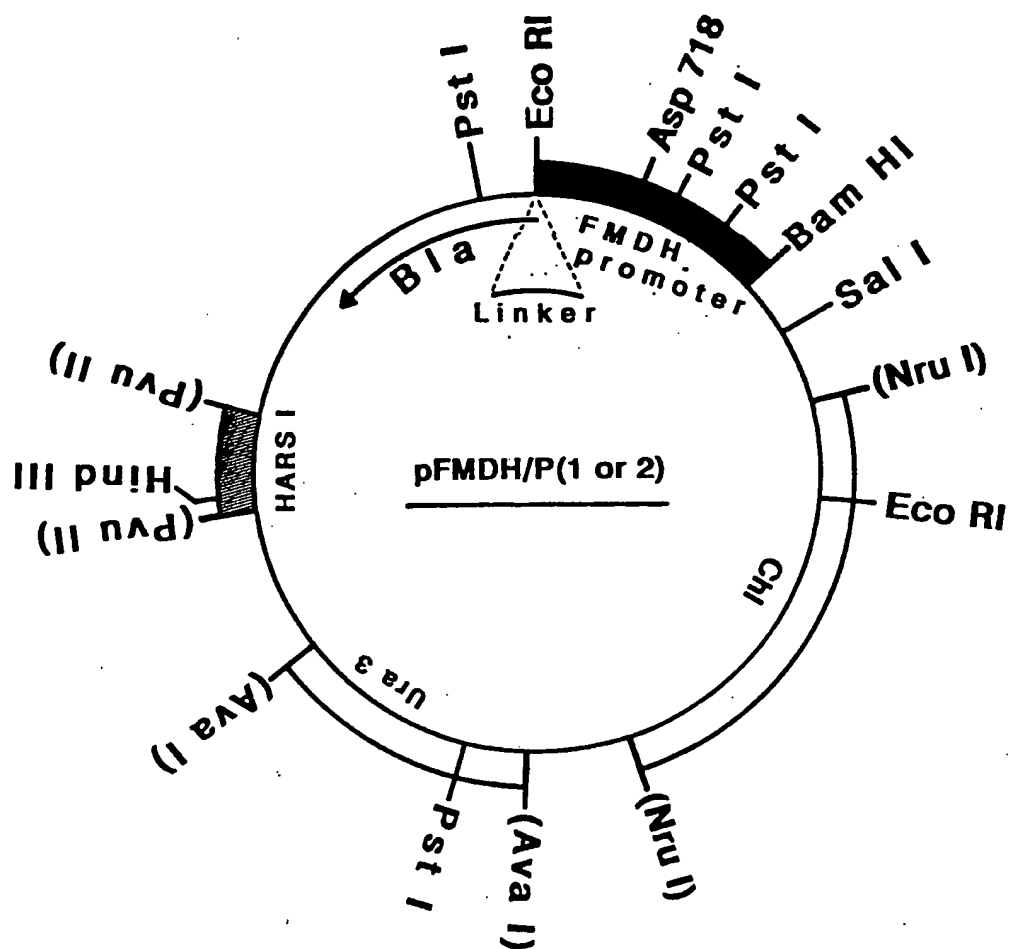
      2230      2240      2250      2260      2270
CCAACGTCTACACGGGAACGTTGATGTGCGACCATCGTGTTCTCAACAGACTXCAA

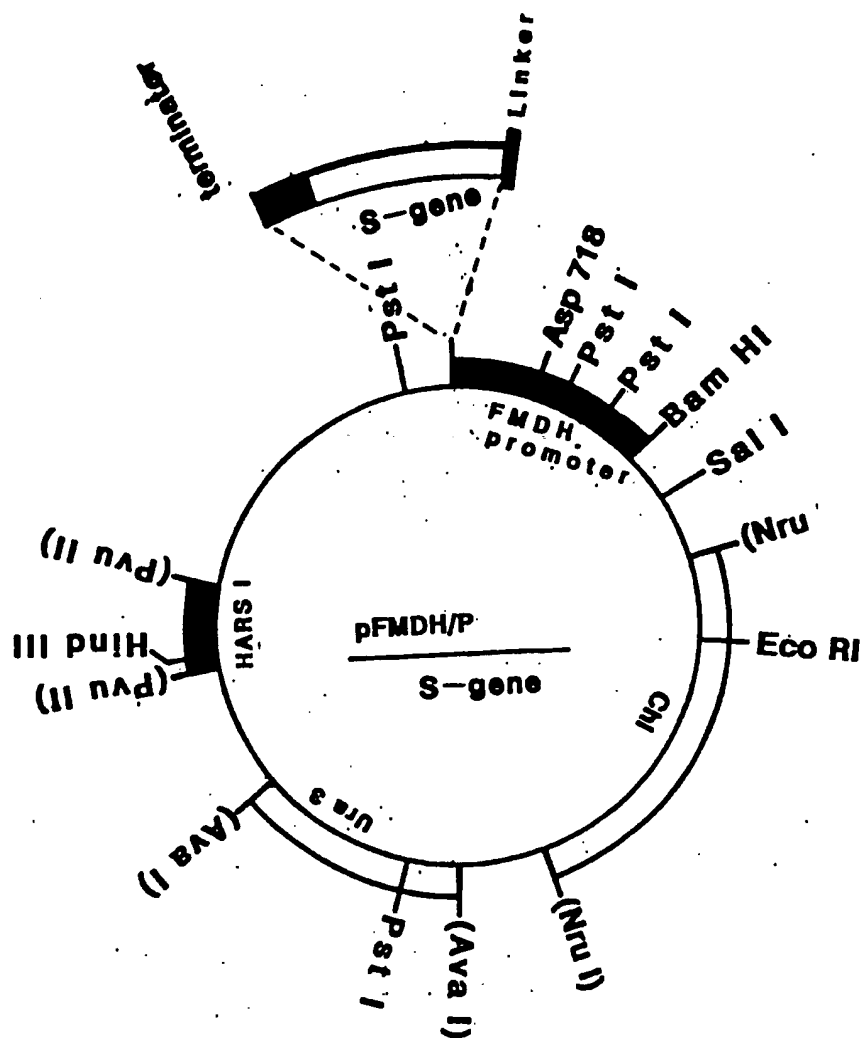
```

F

FIG.5c Nucleotide sequence of FMDH gene and its 5',3' control regions.

FIG.6 Plasmid containing the fusion of β -lactamase gene with FMDH promoter.





Linker :

5'-NcoI-BamHI/EcoRI (blunt ended)-3' (in B version)

Fig. 7 Plasmid containing the hepatitis S-gene

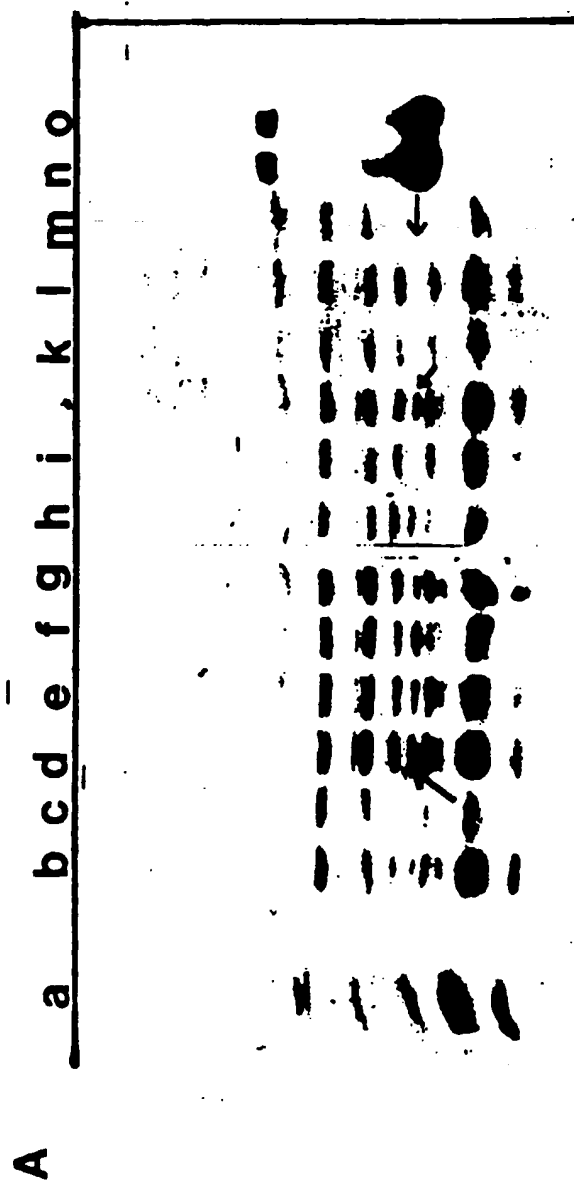


Fig. 8 Western blot-stained by peroxidase/protein A method

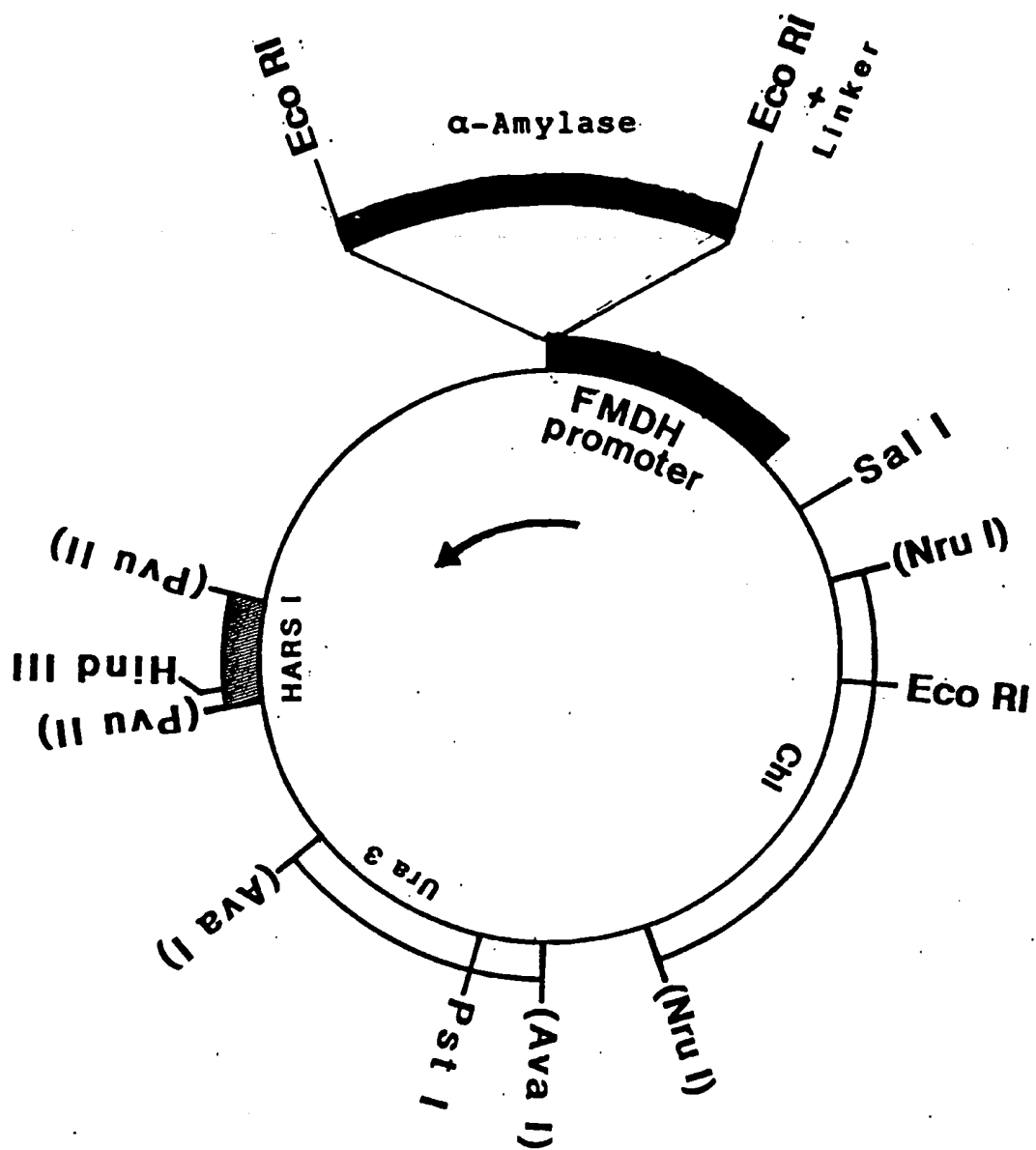
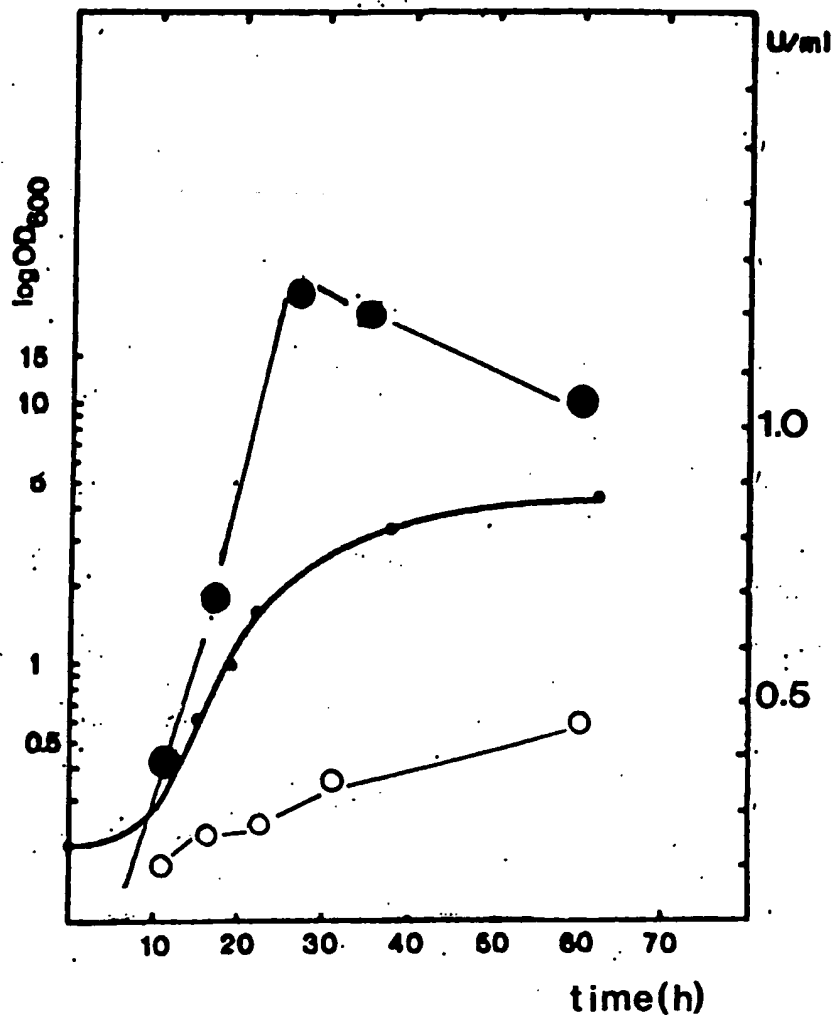


Fig. 9 Plasmid expressing α -amylase gene

Growth on YNB-medium with 1% methanol



- — Cell density
- — Intracellular α-amylase activity
- — α-amylase activity in medium (secretion)

Fig 10 Growth of transformants on medium containing methanol (induction)

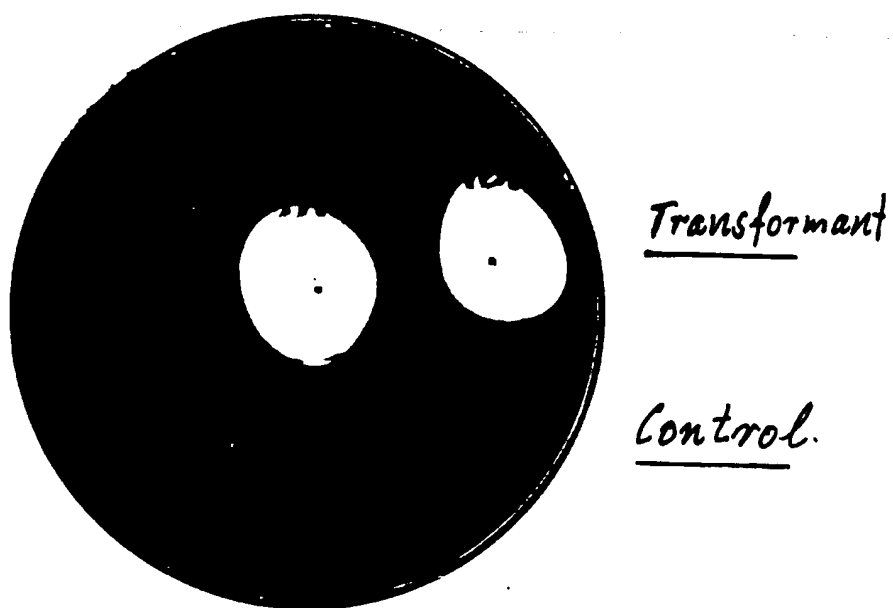


Fig.11. The formation of halo after applying on the plate 50 ul of the medium from transformants _upper row- and from control untransformed strain LR9-lower row.

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